#7

CGNE-62-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	
Comai, et al.	Examiner: P. Rhodes
Serial No. 07/431,429	Art Unit: 184
Filed: November 3, 1989	DECLARATION UNDER 37 CFR 1.131
For: FIGWORT PLANT PROMOTER AND USES	

Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Dear Sir:

I, Margaret P. Sanger do hereby declare as follows:

1. I am a co-inventor of the subject application. The work represented in the attached notebook pages was conducted by myself or under my direction and supervision in the United States at least prior to November 13, 1988.

2. Figwort mosaic virus 34S promoter construct pFWP-101 is described in the subject patent application.

3. Photocopies of relevant pages from my experimental notebook are attached hereto as Exhibit A.

4. Electroporation of plant protoplast with pFWP-101 was conducted as shown on notebook pages 67-69 in Exhibit A.

5. Electroporated protoplasts were analyzed and GUS expression confirmed as demonstrated on notebook pages 69-71 in Exhibit A.

DECLARATION

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 11/21/91 By: Margaret Sanger
Margaret P. Sanger

enclosures: Exhibit A

Abstracts of Presentations at the 1988 Annual Meeting of The American Phytopathological Society and the Pacific Division

November 13–17, 1988 • Town & Country Hotel • San Diego, California

The number above an abstract corresponds to its designation in the program of the 1988 APS Annual Meeting in San Diego, CA, November 13–17. If a presentation was not given at the meeting, the abstract is not printed among the following pages.

The index to authors begins on page 1619.

1

INTERACTION BETWEEN ACID RAIN AND DROUGHT STRESS ON FIELD CORN. R. Knittel, E. J. Pell, and D. P. Knievel, The Pennsylvania State University, University Park, PA 16802.

Field and greenhouse experiments were conducted in 1986 and 1987 to test the hypothesis that acid rain could predispose Zea mays (cv B73 X Mo17) to drought stress. Plants were treated with 3.0 cm/wk of simulated acid rain (SAR) of pH 3.0 or 5.0, and ambient rain was excluded. Two droughts were imposed, one following silking and a second following pollination. Effects were determined by physiological and histological parameters and by yield determination. Drought but no pH significantly reduced yield both years. In 1986 there was a drought X pH interaction in which plants treated with rain pH of 3.0 and drought had more kernels/ear and lower stomatal and cuticular conductance. In 1987 there were no interactions and conductance was lower for plants treated with rain of pH 5.0. Scanning electron microscopy shows no correction between presence of plate-like epicuticular wax formations and rain pH on foliage of greenhouse grown plants treated with SAR of pH 3.0.

2

INFLUENCE OF O_3 AND NITROGEN ON GROWTH AND PARTITIONING OF ASSIMILATE IN RADISH PLANTS. E. J. Pell and C. Vinten-Johansen. The Pennsylvania State University, University Park, PA 16802.

Raphanus sativus L. 'Cherry Belle' were grown with suboptimal, optimal and supraoptimal soil nitrogen (N). Plants were treated with charcoal filtered air or O_3 levels averaging 38 or 66 ppb delivered from 1000 to 1930 h for 32 d. Dry weight and total nonstructural carbohydrate (TNC) were measured as determinants of growth and assimilate. 66 ppb O_3 induced significant reduction in weight of hypocotyls and roots, and root/shoot ratio while elevated N resulted in increased weight of all plant parts. Effects of O_3 were more apparent at the optimal and supraoptimal N treatments. TNC levels were reduced at higher levels of N but lower in hypocotyls and roots harvested from plants treated with 66 ppb O_3. Elevated O_3 levels increased TNC content of foliage in the supraoptimal N treatment.

3

GAS EXCHANGE RESPONSES OF SOYBEAN CULTIVARS TO SHORT TERM EXPOSURE OF SULFUR DIOXIDE AND OZONE. Wen S. Sheng and Boris Chevone. Dept. of Plant Pathology, Physiology and Weed Science, VPI & SU, Blacksburg, VA 24061.

Soybean cultivars 'Dare', 'Williams 82' and 'Essex' were exposed to 0.7 ppm sulfur dioxide (SO_2), 0.2 ppm ozone (O_3) or filtered air for 4 hr in environmentally controlled fumigation chambers. Gas exchange measurements were taken at intervals of 30 min during the fumigation. Both O_3 and SO_2 resulted in a reduction in net photosynthesis (Pn) and stomatal conductance (Cs). All the cultivars developed typical SO_2 or O_3 symptoms during or after fumigation. Suppression of Cs and Pn by SO_2 occurred primarily within the first 60 min of exposure. However, dramatic effects of O_3 on Cs and Pn did not occur until 90 min after the fumigation was initiated. Control plants exposed to filtered air maintained consistent Pn and Cs throughout the 4 hr period.

4

RESPONSE OF FIELD-GROWN LOBLOLLY PINE TO OZONE OVER THREE GROWING SEASONS. S. B. Shafer and A. S. Heagle, USDA/ARS, N. C. State Univ., Dept. of Plant Pathology, Raleigh, NC 27695-7616.

Seedlings (4-mo-old) of Pinus taeda were planted in a field and exposed daily during 3 growing seasons (May 27-Oct. 24, 1985; Apr. 8-Oct. 16, 1986; Apr. 8-Oct. 1, 1987) to charcoal-filtered (CF) air, nonfiltered (NF) air, or NF air supplemented (12 hr/da) with O_3 to produce O_3 concentrations in proportions of 1.25, 1.50, 1.75, or 2.00 x NF in open-top chambers (128 seedlings/chamber). Plants were harvested at the end of each growing season (4 families in 1985 and 1986; one of the 4 in 1987). Significant dose-response models (seasonal 12-hr/da mean O_3 concentrations vs. above-ground biomass components) developed for plants harvested in Oct. 1985 were linear. After remaining plants were exposed in subsequent years, data for some family-variable combinations were fitted by Weibull models that indicated a level of O_3 near 1.25 x NF was required for noticeable plant responses. However, data for one family continued to indicate linear dose-response relationships. Depending on the year-family-variable combination, models predicted yields in NF air (3-season average of the 12 hr/da seasonal mean O_3 concentrations=0.048 µl/l) that were suppressed as much as 21% from those predicted for CF air (3-season average O_3=0.025 µl/l).

5

OZONE EFFECTS ON LEAF CARBOHYDRATE CONTENT OF DIFFERENTIALLY SENSITIVE RADISH POPULATIONS. C.T. Gillespie and L. D. Moore. Dept. of Plant Pathology, Physiology, and Weed Science, VPI & SU, Blacksburg, VA 24061.

Three populations of radish, Raphanus sativus L. cv 'Cherry Belle', previously selected for differential sensitivity to ozone were exposed to either filtered air or 0.10 ppm ozone for 4 hr/day, 3 day/week, for 3 weeks. At intervals of 5 days, one leaf from each of the first two leaf pairs was harvested and analyzed for sugar and starch levels. Exposure to ozone increased the free sugar level in leaves in all populations as compared to filtered air treated plants, but to a lesser degree in the ozone resistant population than in the ozone sensitive or non-selected populations. Leaves from these two populations exposed to ozone also had elevated starch levels. Increases in the leaf carbohydrate pool may be a product of decreased carbon translocation to sinks such as the roots and hypocotyl and could account for the greater sensitivity of below ground parts to ozone.

6

RELATIONSHIP BETWEEN COMMON ROOT ROT AND WINTER WHEAT FORAGE PRODUCTION. J. T. Mathieson, C. M. Rush, and K. B. Porter. Texas A&M University, Texas Agricultural Experiment Station, Bushland, Texas 79012.

Both Imazalil treated and untreated seed of seven winter

Camera-ready abstracts are published as submitted. The abstracts are not edited or retyped in the APS headquarters office.

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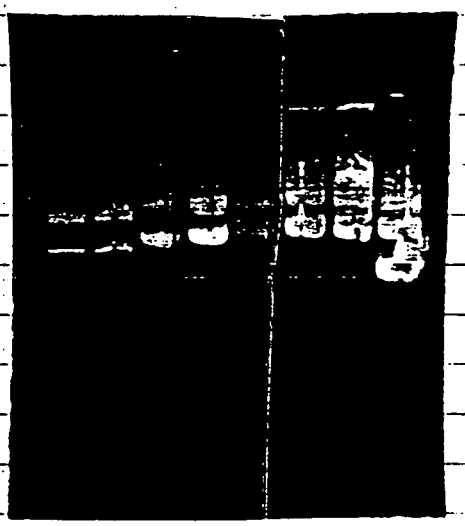
Next need to prepare enough DNA to test promoter region by transient expression system using GUS activity

- inoculated 50ml LB (Cm 5ug/ml) with pCGN7304-101)

- harvested - 7304-101 (50ml) & extracted plasmid DNA

- Meanwhile to Sal I cut FMV-X2 & FMV-X4
  - phenol/chloroform extracted
  - EtOH ppted.
  - dried & resuspended in 20ul for next infection test on Datura stramonium

① FMV (Sal I cut) X2 - 1ul
② FMV X4 (Sal I cut) - 1ul
③ pCGN 7304-101 uncut - 1ul
④ pCGN 860 (35S-HM-tml3') 0.5ul
⑤ pCGN 852 (35S-LM-tml3') 0.5ul
⑥ pCGN 844 (mas3'-HL-Ocs3') 0.5ul
⑦ 7304 (A) - 1ul
⑧ 7304 (B) - 1ul



Checked concentration via A260

CGN 7304-101 250x dil = .273 --- 2.3mg/ml
CGN 7304 250x dil = .914 ---- 9.14mg/ml
CGN 3000 2500x dil = .5Ox ---- 50mg/ml
CGN 860 1000x dil = .332 ---- 13.5mg/ml
CGN 852 5000x dil = .274 ---- 54.6mg/ml

Need to check CGN 7000 or get

- Electroporation of FMV promoter sequence-GUS into tobacco protoplasts:

- Want to test out FMV full length RNA promoter region - to see if altered DNA conf. works
  - If AUG's cause problems?
  - Generally - is there any promoter activity

will test the following constructs for comparison w/ FMV:

(A) CGN 7000 (mas 5'-GUS-mas 3') -- 50µg + 175µg pUC19 DNA
(→ may have combined into (A) by mistake - assays will tell.
(B) CGN 7304 (double 35S-GUS-Mas 3' -- 50µg + 175µg pUC19

(C) CGN 7304-101 (FMV-GUS-mas 3') -- 50µg + 175 µg pUC19

(D) pUC19 - no GUS 225µg DNA.

The protocol used was that of Bruby Jones & is outlined in my Lab book #5208-002-E, 59+60

In brief:
- 20 Xanthi leaves / 30 mls Enzyme Solution
- (- 17 Xanthi leaves / 30 mls Enzyme "
  - → infiltrated at 300 mtorr ~10.40 A
  - incubated in dark ~ 2 hrs → 1:15 P
  - The material was agitated by running up & down wide bore (cut Japanese pipettes & then narrower (normal) bore Japanese pipettes
  - strained through 53 um filter
  - protos were centrifuged out of Enzyme ~ 7 PM & washed 2X (#2 setting IEC Clinical centri for 4.5 min each spin)
  - counted protos - counted 3 sectors & averaged.

protoplast count @ 416
@ 415 $\bar{x} = 453 (\times 10^4/ml)$
@ 529

∴ have 8mls at 4.5×10^6 protos/ml
$= 36 \times 10^6$ protos

If electroporate 9 samples at 3.5 million/ml
then need $9 * 3.5 = 31.5 \times 10^6$ protos

- collected, spun → suspended in ELECTROPORATION BUFFER
- Added DNAs, ↓ → 1ml/sample
- Electroporated ~60msec
  ~1200 μfarrads
  ~225 μg Total DNA/sample

Following electroporation 1ml/sample were added to 10ml culture medium in plant culture petri dishes (25×100 mm)

- Put to incubate in dark @ 25°C for ~2days.

Checked protos in ?M — Still looked OK.

- Protos - O.K. - no microbial contamin.
  - protos still intact & looking O.K.

So - need to harvest, extract, & assay for GUS activity

Collected protos by pipetting them into 15ml screw-cap centrifuge tube, rinsing out plate w/ 3mls wash buffer → tube & centrifuge for 8min at ½ full (~500 rpm) for 8min. (note had to resuspend 1 sample because disturbed pellet & found 4min at ¾ full is also O.K.).

M.S

- pipetted off supernatant & resuspended protos in 1ml Extraction Buffer;

GUS Extraction Buffer*

50mM Na_2HPO_4 (pH=7.0 w/ NaH_2PO_4)
10mM βME (14.4M sat) = .7ml/l
10mM Na_2EDTA
0.1% Sodium Lauryl Sarcosine
0.1% Triton X-100
→ Plus 1uM PMSF mw=174.2 should use ~100uM ~20mg/ll

- put in sonic bath for 2 X 60sec & checked for protoplast breakage -- poor - tried some more still at least 80% intact
- polytroned each sample for ~5sec each - most proto's were broken & rest looked disrupted.

- put into 1.5ml conical cent. tubes & µfuged for 4min in cold. Used supernatant as crude enzyme soln.

GUS Assay:

MW~440

- used 100ul Enzyme prep
- add 400ul of Assay buffer - (Extraction buffer w/ MUG 1.25mM)
  - T 500ul
  - 125µl 100mM MUG → 10ml ext. Buff.
  - so final assay conc of 500ul vol = 1mM
  - for 10ml use 125ul of a 100mM MUG soln.
- at 37°

Incubate for various times

[signature]

MUG = methylumbelliferone glucuronide (4-methyl umbelliferyl β-D-glucuronide MW~440 352.3

* Richard A Jefferson (1987) "Assaying Chimeric genes in plants: the GUS gene fusion system." Plant Molec. Biol. Reporter V5 #4 PP 387-405. (

m.s.

Stop Buffer

Stop reaction w/ 900ul of 0.2M Na_2CO_3 (21.2g/L)
- this stops rx as well as ↑ fluorescence of methyl umbelliferone

To correlate relative fluorescence with [MU] set-up standards (excit = 365, emiss. = 455)

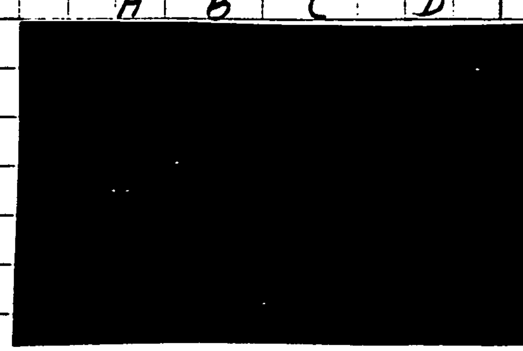
Jefferson recomends following conc range:

			# of 100uM stock
100uM =	0.1uM	(5ul 10uM + 495 → 500ul)	5 + 495
500uM	0.5uM	(25ul 10uM + 475 Ext Buf.)	25 + 495
	1.0	(5ul 100uM + 495 EB)	50 + 450
	2.0	(10ul 100uM + 490)	100 + 400
	4.0	(20ul 100uM + 480)	200 + 300
	8.0	(40ul 100uM + 460)	400 + 100

Added substrate to extracts at 3:30 PM
- took first time pts @ 1hr 15min
- took 2nd time pts @ 2hr 45min

1st looked at samples on UV transilluminator
(A) = CGN 7000 + CGN 7304 50+50+175 pUC DNA
(B) = rinsed pipette tip of CGN 7304 (i.e. mainly no DNA) (175 pUC)
(C) = CGN 7304-101 (50ul + 175 pUC)
(D) = pUC19 (225 ug)

A B C D



Thus FMV-promotor region is active and may be so to a level similar to a single 35S of CaMV

Fluorometric assay → HMD

m.S